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REVIEW

Proteomics and low-temperature studies: bridging the gap between gene expression and metabolism

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Exposure of plants to low, non-freezing temperatures results in genetically programmed changes in the physiology and biochemistry of plants that are critical for low-temperature survival. While genomic and transcript-profiling studies have provided a wealth of information about the process of cold acclimation, there is growing recognition that the abundance of mRNA transcripts is not always representative of cognate protein levels and that mechanisms of post-translational regulation must also play an important role. Recent advances in proteomic technologies have greatly increased the utility of studying global changes in proteins and significantly increased the efficiency and reliability of bidimensional gel electrophoresis (2DE). This has been accompanied by advances in mass spectrometry (MS) and in protein-sequence databases used in the identification of separated proteins. Although encumbered with its own constraints, proteomics has become a powerful method used to study the relationship between gene expression (transcriptomics) and metabolism (metabolomics). New techniques in gel-based approaches, such as difference gel electrophoresis (DIGE), are now available to provide both qualitative and quantitative data about the differential expression of proteins. Following 2DE separation and analysis, selected spots are usually subjected to tryptic digestion and identified using electrospray ionization, matrix-assisted laser desorption/ionization-time of flight-MS and/or tandem MS. In this review, the advantages of the DIGE technique over existing 2DE techniques will be presented, and the utility of 2DE techniques in low-temperature studies as a complement to transcriptomics, genomics and metabolomics will be discussed. Examples of proteomic studies utilizing different tissues or subcellular compartments in response to cold will also be presented.

Abbreviations – 2DE, bidimensional electrophoresis; AFP, antifreeze protein; BN-PAGE, blue native-polyacrylamide gel; Clp, caseinolytic protease; DIGE, difference gel electrophoresis; ESI, electrospray ionization; HSP, heat-shock proteins; LEA, late embryogenesis abundant; MALDI, matrix-assisted laser desorption/ionization: MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAGE, polyacrylamide gel; PR, pathogenesis-related; PTM, post-translational modification; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel; TOF, time of flight.

Introduction

Exposure of plants to low temperature influences the productivity, as well as the geographical distribution, of both wild and cultivated plants and induces significant changes in plant metabolism (Alberdi and Corcuera 1991, Graham and Patterson 1982, Guy 1990). The response of a plant to cold stress can be complex and will depend on whether the temperature remains above or below 0° C, the duration of the exposure and whether or not the plant is capable of cold acclimation. In the case of woody plants, the response to low temperature is also associated with the response to short photoperiod. The process that is induced in plants in response to low, non-freezing temperatures and allows them to increase their freezing tolerance is referred to as cold acclimation (Guy 1990, Thomashow 1998). It involves distinct changes in gene expression, protein expression and metabolites (Hurry et al. 1995, Kaplan et al. 2004, Orvar et al. 2000, Öquist et al. 2001, Renaut et al. 2004, 2005, Thomashow et al. 2001).

Environmental stresses that result in cellular dehydration, such as freezing, salt and water stress, often lead to similar changes in plant gene expression and metabolism (Cook et al. 2004, Kaplan et al. 2004, Kreps et al. 2002, Rabbani et al. 2003, Seki et al. 2001), and there exists cross-talk in their signalling pathways (Chinnusamy et al. 2004, Knight and Knight 2001). It has been demonstrated that these dehydrative conditions can trigger both common response pathways [e.g. Ca^{2+}-mediated response, mitogen-activated protein kinase (MAPK) cascades (Zhang and Klessig 2001), as well as stress-specific ones (e.g. ICE1, Chinnusamy et al. 2003, Fowler and Thomashow 2002, Sung et al. 2001)]. The use of genomic approaches, such as microarrays and the bioinformatics analysis of expressed sequence tags compiled from different stressed and non-stressed plant species, as well as different tissues within the same species, have provided a wealth of information by revealing global changes in gene expression and by revealing the complexity of the response. While low-temperature-inducible genes have been identified and used to improve the freezing tolerance of plants by gene transfer (Holmberg and Bulow 1998), the increased tolerance is often very modest and can result in deleterious phenotypic traits. It has been recognized that the mechanisms of coping with low-temperature stress are complex and multigenic (Hughes and Dunn 1996, Thomashow 1998). Therefore, to better understand the process of cold tolerance and develop strategies to improve resistance, it was found that the genomic approaches need to be complemented by qualitative and quantitative analyses of the plant at several levels including the transcriptome, proteome and metabolome.

At the transcriptome level, powerful techniques like microarray analysis exist and provide a wealth of information about which genes are involved in environmental-stress response and adaptation. These results, however, must be balanced by the fact that mRNA abundance and protein level are not clearly correlated (Gygi et al. 1999), that low-copy number mRNAs (potentially very important for regulation) are not measured as readily as abundant mRNAs and that gene expression studies do not provide information about either the subcellular localization of gene products or the post-translational modifications occurring to a protein that may be essential for its function, transport and activation (Rose et al. 2004). In response to cold stress, a broad number of genes have been shown to be either overexpressed or repressed (Chen et al. 2002, Fowler and Thomashow 2002, Kreps et al. 2002, Seki et al. 2001, Shinozaki et al. 2003, Sung et al. 2001, 2003).

In contrast to transcript profiling, metabolomics can be used to provide a global profile of a wide array of metabolites (Cook et al. 2004, Gray and Heath 2005, Kaplan et al. 2004). This information is necessary to understand the biology of a cell or an organism, because the levels of various metabolites are the net result of direct enzymatic activity and of indirect cellular regulation over transcriptional or biochemical processes. Proteomics, the study of global changes in proteins, provides an essential bridge between the transcriptome and the metabolome. Through global studies of changes in proteins in response to low temperatures and other environmental stresses, novel proteins, protein–protein interactions and post-translational modifications can be identified. Metabolomics, along with proteomics and transcriptomics, and their integration into system biology, will provide a comprehensive analysis of cell biology and the development of strategies to alter cellular metabolism in a directed and controlled manner.

Analysis of large portions of the proteome in response to specific treatments is now possible through techniques such as bidimensional electrophoresis (2DE) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). These tools can provide a more direct assessment of biochemical processes by providing information about the actual proteins involved in the enzymatic, regulatory and structural functions encoded by genes and regulated by transcription factors. Different families of proteins are known to be associated with a plant's response to cold stress by being newly synthesized, accumulating or decreasing. Among other things, these proteins are involved in

signalling, translation, host-defence mechanisms, carbohydrate metabolism and amino acid metabolism. These proteins include dehydrins and other late-embryogenesis-abundant (LEA) proteins (Close 1997, NDong et al. 2002, Welling et al. 2004, Wisniewski et al. 1999) and also antifreeze proteins (AFPs) (Griffith and Yaish 2004), heat shock proteins [e.g. heat-shock protein 70 (HSP70); Sung et al. 2001, Wisniewski et al. 1996] and other cold-regulated (COR) proteins (Jaglo-Ottosen et al. 1998).

Although the feasibility and the reproducibility of bidimensional gels have been the subject to a large debate, it remains a commonly used method to separate proteins from complex mixtures. This review will discuss the potential of using bidimensional electrophoretic techniques followed by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) or MS-MS analyses to conduct proteomic analyses of plants. Recent improvements of gel-based technique by the use of multiplexed gels with fluorescent labelling, referred to as two-dimensional difference gel electrophoresis (2D-DIGE), will also be discussed. Among its multiple advantages, DIGE broadens the dynamic range of the analysis by detecting more low abundant proteins without saturating the highly abundant ones and provides quantitative results. While gel-free techniques are also available for performing proteomic analyses, they are not currently widely used in the field of plant biology. This is due to the fact that gel-free techniques require that the sequence of the entire genome of the organism to be examined be available. A compilation of existing proteomic results related to cold stress in plants will also be presented.

Proteomics: a novel approach to unravel response to stress

Proteomics is becoming a powerful tool to analyse biochemical pathways and the complex response of plants to environmental stimuli. The development of this technique is, at least partly, dependent on genome-sequencing projects which provide essential data on the specific amino acid sequence of proteins coded for by the genes of an organism. This data can be used to identify proteins in proteomic studies. Current proteomic techniques also allow for expression analysis of a large number of proteins on a single gel (Park 2004, van Wijk 2001). 2DE, as a technique for the study of proteins, has been used for more than 30 years (O'Farrel 1975), and substantial improvements have been made over that period of time. Gel size has increased, resolution or the number of spots that can be independently observed has increased and the ability to define a pH gradient has greatly improved. In fact, the pH gradient can now be narrowed down to 1 pH unit on a 24-cm gel (Fig. 1).

So, what is a proteome? It can be defined as the full complement of proteins expressed by the genome of a cell, a tissue or an organism at a specific time point (Aebersold and Goodlett 2001, Wilkins et al. 1995). 2DE analysis of the proteome requires a gel-based separation of the extracted proteins (with or without prefractionation of the sample). After 2DE, selection and isolation of spots of interest, they are submitted to tryptic digestion and identification of the digested protein using MS while searching in public databases using computer software programs.

If the genome of the plant being studied has been sequenced, peptide mass fingerprinting is usually sufficient to identify a protein. When such genomic data do not exist, sequencing of the peptide by MS/MS may be required to obtain the identification of a protein of interest (Hirano et al. 2004). Basically, MS analysis consists of an ionization source, a mass analyser and an ion detector. MS analysers are measuring the mass-to-charge ratio (m/z) of either intact or fragmented peptides. Prior to their analysis, proteins or peptides need to be ionized. The m/z measurement can be based, for example, on the flight time of the analytes through a TOF analyser tube. Common types of MS analysers are the MALDI and the electrospray ionization (ESI), which analyses the mass of intact charged peptides (Aebersold and Goodlett 2001). In short, in the case of the MALDI, the energy of a laser is absorbed by the matrix and causes the ionization of the analytes. The ESI system ionization is based on a high electric potential applied to a capillary needle in which the sample is passing through. This produces a mist of highly charged droplets. After evaporation of the solvent, ions will reach the detector of the mass spectrometer (e.g. TOF or quadrupole) (Gaskell 1997). Tandem MS/MS is often used to increase either the number of positive identifications or the reliability of the results obtained by MS (Liska and Shevchenko 2003) and consists of an ion generator, a first mass analyser, a collision cell, a second mass analyser and an ion detector. During MS/MS, the first mass analyser is used to select a particular peptide sent in a collision cell where it is fragmented. The resulting fragment ion spectra allow the determination of the peptide sequence by the mass of the amino acids. Presently, cross-species identification of proteins can be made due to technical improvements in MS.

In addition to 2DE, another technique, referred to as blue-native PAGE (BN-PAGE) (Schagger and von Jagow 1991), has been developed to study hydrophobic and/or membrane proteins. Association of proteins in

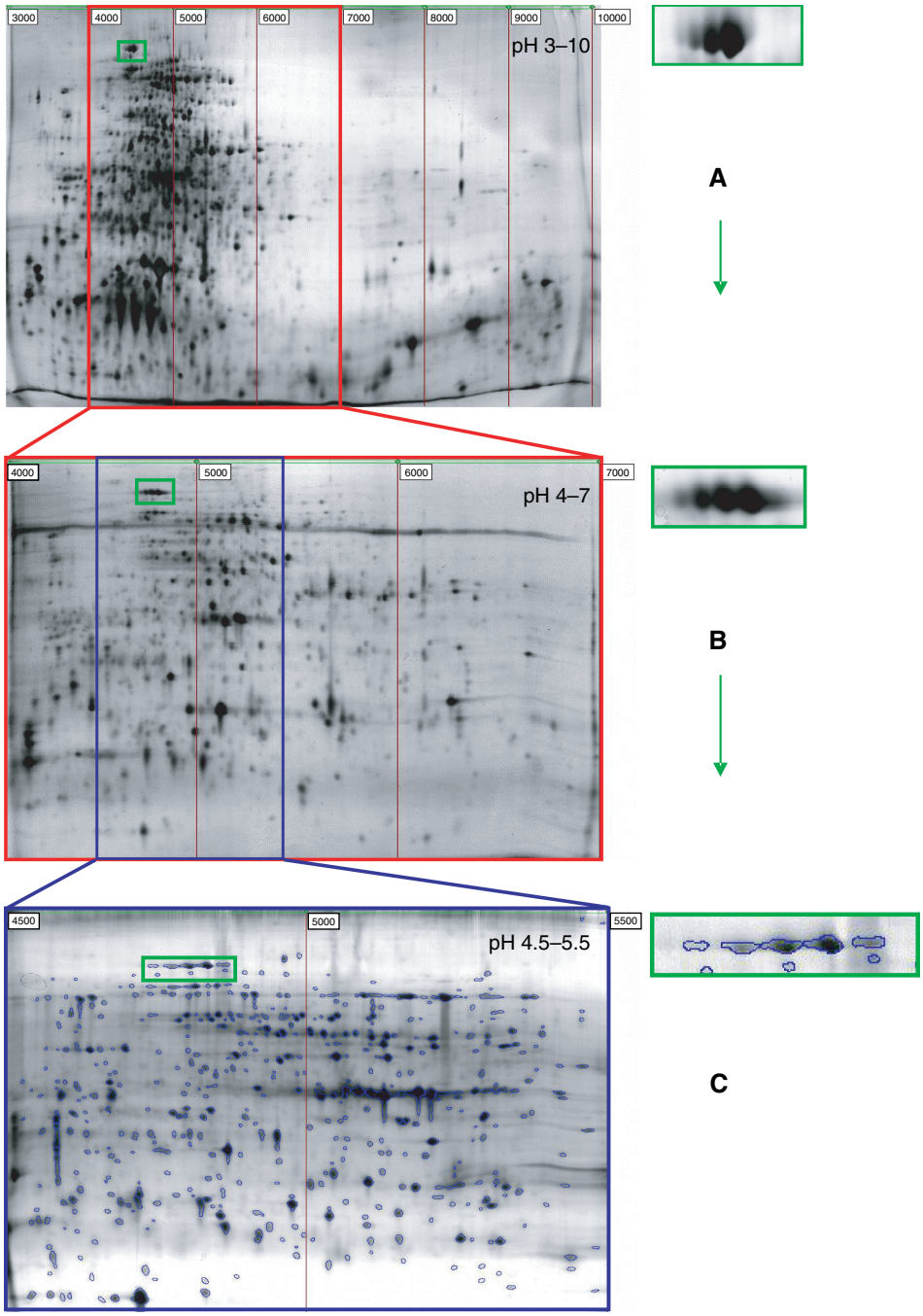

Fig. 1. Improvement in the separation and resolution of proteins as a result of narrowing the pH gradient on 24-cm gels, stained with silver nitrate. (A) Separation of total proteins from poplar leaves was done between pH 3 and 10 (1109 spots). (B) pH 4–7 (1059 spots). (C) Maximal separation currently available is 1 pH unit (presented here: 4.5–5.5) allowing the separation of 573 spots.

complexes involved in photosynthesis or respiration, for instance, could be analysed using this technique. It involves a double separation of native proteins using a mild detergent for solubilization. In the 'first' dimension, 'super-complexes' extracted from mitochondria or chloroplasts are separated according to their mass on a gradient native polyacrylamide gel, resolving complexes of mass ranging from 700 kDa down to 100 kDa. In the second dimension, a classical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins associated in complexes are then separated according to their mass (Fig. 2). As examples,

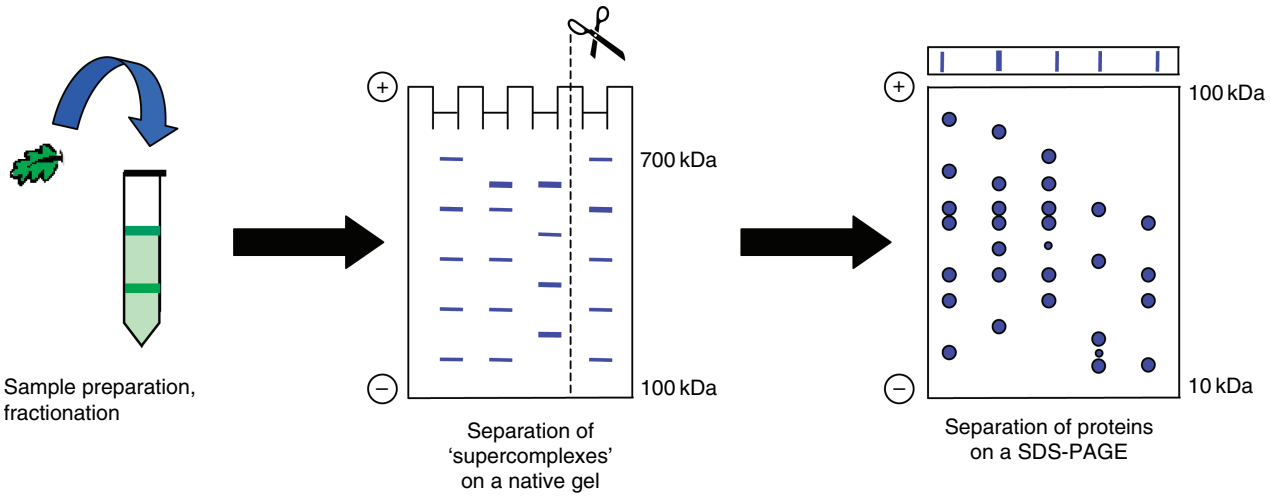

Fig. 2. Diagrammatic illustration of the blue-native polyacrylamide gel (PAGE) technique. Mitochondrial, plastidic or membrane proteins are extracted and fractionated prior to separation on a native PAGE. Lanes are then cut and submitted to further separation using sodium dodecyl sulphate-PAGE resulting in separation of protein complexes.

this technique has brought new insights on the composition and the regulation of different complexes and/or super-complexes in plastids and mitochondria, such as in the respiratory chain of mitochondria (Eubel et al. 2003) or the caseinolytic protease (Clp) machinery (Peltier et al. 2004). By revealing the composition of the Clp complex, and its association with chaperones, it suggested that the regulation of the proteolytic activity of the machinery could be done through association with chaperones and recognition of the substrate in the three-dimensional structure instead of a regulation through the expression of the corresponding genes.

The field of proteomics is expanding rapidly and new approaches and protocols are constantly being developed. This has led to the appearance of several new journals dedicated to these technologies (e.g. Proteomics, Molecular and Cellular Proteomics, Journal of Proteome Research).

Proteomics: problems and pitfalls

While the genome of an organism is a relatively fixed entity, the proteome (similar to the transcriptome) is dynamic. Therefore, there are potentially many proteomes present in an organism, and the picture of the proteome that one is presented at any single point in time will depend on many factors including developmental stage, response to biotic and abiotic stress, the organ or tissue being examined or even the cellular compartment being studied. Moreover, it has been estimated that an organism has at least one order of magnitude greater number of proteins than genes (Pennington and Dunn 2001, Service 2001). Another complicating factor is that there is no way to increase the amount of low-abundant proteins as one can do with low-copy genes using the polymerase chain reaction (PCR).

Elucidating the amino acids sequence of a protein is not enough to understand its function or its interactions with other proteins and macromolecules. The acquisition of biological activity often is only accomplished when a protein has been located in the appropriate compartment and/or has undergone a post-translational modification (PTM). PTMs must be considered to understand the dynamics of the proteome. Fortunately, MS allows elucidating the site and nature of many PTMs (Mann and Jensen 2003). Extraction, separation and modification of the plant proteome can be dramatically altered by the presence of interfering substances such as phenolic compounds, pigments, nucleic acids, proteases, carbohydrates, lipids and so on (Newton et al. 2004). In addition to these considerations, results need to be quantitative and amenable to statistical analyses to differentiate real biological variations (both large and small) from artefacts due to technical preparation or random variation.

Advances in proteomic technology have added new levels of complexity in sample preparation, data analysis and the instrumentation used to obtain high-throughput analysis. Despite this complexity and the restraints associated with proteomics the interest in applying this technology to plant biology has greatly increased in recent years. Current techniques can now compensate for some of the problems through increased sensitivity, reproducibility and resolution. It is important to remember, however, that current proteomic studies represent only a small part of the complete proteome and protein

interactions existing in an organism due to the problems and limitations described.

2D-DIGE: A recent advance in 2DE technology

DIGE (Ettan™ DIGE system, GE Healthcare, Buckinghamshire, UK) technology is a significant advance in proteomic technology that addresses several of the problems encountered in the reliability, quantitative analysis and efficiency of proteomic research. In this method, proteins are extracted from different samples and labelled with one of three different fluorescent dyes (Fig. 3). The three differentially labelled samples can then be combined and separated using 2DE on a single gel. Using a laser scanner, we detected proteins labelled by each of the dyes separately using the excitation and emission wavelength corresponding to the specific dye. The resulting images are then analysed using proprietary software (DeCyder, GE Healthcare) that allows differentially expressed proteins to be rapidly detected and quantified. DIGE has several advantages over existing 2DE technology: it improves reproducibility by reducing gel-to-gel variability, reduces the number of gels needed to separate samples, has a wide dynamic range that allows for the detection of low abundance proteins (at least 4–5 orders of magnitude) while avoiding saturation by high-abundance proteins and is designed to directly accommodate subsequent MS analysis. Moreover, the use of an internal standard (i.e. a proportional mixture of all the samples used for the experiment) run on every gel allows a better matching across the different gels and a more accurate statistical analysis across the multiple gels by the normalization of individual samples compared with this internal standard. DIGE also provides the advantage of being able to do a quantitative analysis of the derived protein spots, as the intensity of the spot detected by the scanner is proportional to the amount of protein in the gel. DIGE provides a dynamic and quantitative aspect to 2DE analysis that allows rigorous statistical analyses of changes in protein abundance that reflect biochemical changes in an organism. Studies utilizing this technique have been initiated to examine the expression of total soluble proteins in poplar (*Populus euphratica*) and peach (*Prunus persica*), in

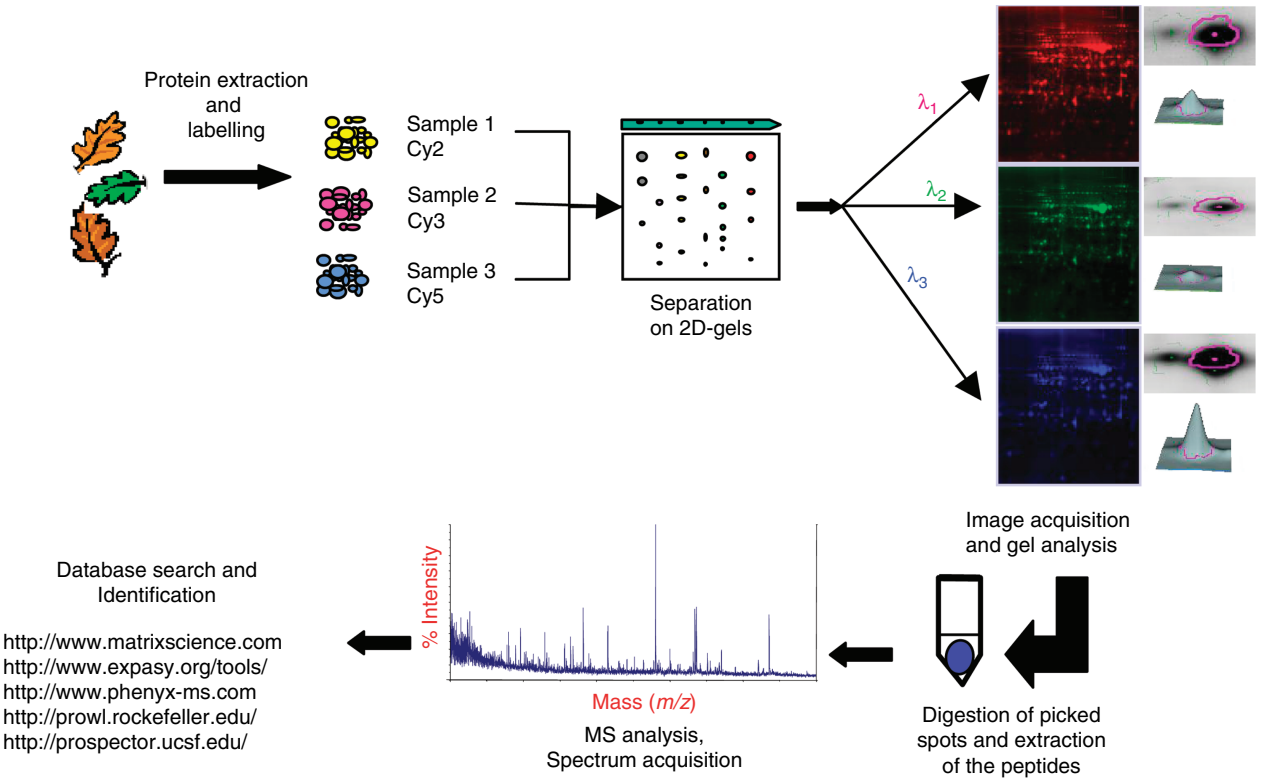

Fig. 3. Illustration of the difference gel electrophoresis method applied to plant tissues and followed by mass spectrometry analysis. After extraction of proteins from different samples, they are labelled with Cy dyes, then mixed and separated on bidimensional gels. After acquisition of images using a multiple-laser scanner, proteomic patterns are analysed and proteins of interest are picked and digested using trypsin. Determination of the mass of individual peptides and, if necessary, sequencing are performed on a mass spectrometry (MS) analyser. The results obtained are then entered into various database search engines to obtain identification of proteins.

response to either drought or short photoperiod and low temperature (M-B Bogeat-Triboulot et al. personal communication, J. Renaut et al. personal communication, respectively). A diagrammatic summary of the peach study is illustrated in Fig. 4. The analysis utilized 15 gels (five triplicates) and a two-way ANOVA to analyse the effect of photoperiod and low temperature on protein expression.

Application of proteomics to low-temperature research in plants

Comprehensive proteomic studies of the cold response of plants using 2DE will provide new information on proteins that play an integral role in freezing tolerance and freeze recovery. This information would greatly complement the data arising from genomic studies and increase our understanding of protein function in specific cellular compartments. Identification of new proteins can be done through gel analysis (1DE, 2DE or BN-PAGE) and subsequent MS analysis while the study of protein–protein interaction will require, for example, the use of a yeast two-hybrid system (Uetz et al. 2000). Detection of post-translational modifications (PTM) can be performed using MS after enzymatic digestion. The mass of the theoretical peptide is compared with the observed mass, and a PTM can be identified (Seo and Lee 2004). Most common PTMs are phosphorylations, glycosylations, cleavage and carboxylations. These modifications are involved, for instance, in the control of metabolic processes and signal transduction, molecular targeting, activation of proenzymes and binding with other molecules (e.g. nucleic acids, Ca^{2+}).

Batch No.	Cy2	Cy3	Cy5
1	Pooled internal standard	Control 1	SD 5°C 3 weeks
2	Pooled internal standard	SD 25°C 3 weeks	SD 5°C 5 weeks
3	Pooled internal standard	SD 25°C 5 weeks	LD 5°C 3 weeks
4	Pooled internal standard	LD 5°C 5 weeks	Control 2
5	Pooled internal standard	LD 25°C 3 weeks	LD 25°C 5 weeks

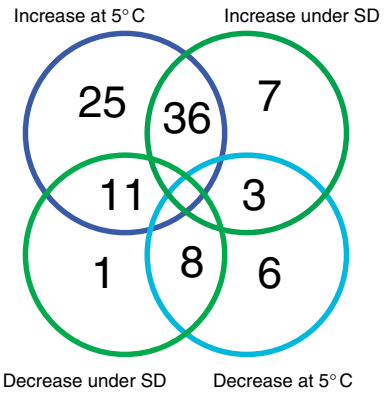

Fig. 4. Difference gel electrophoresis experiment illustrating the effect of short photoperiod (SD) and/or low-temperature on protein expression in peach bark tissues. Trees were exposed to either a short or long photoperiod at either 5 or 25° C. Bark samples were collected at 3 and 5 weeks. A relative proportion of each sample constituted the pooled internal standard, labelled with Cy2 and run on each batch of three gels. Cy3 and Cy5 were used to label the other individual samples. Diagrams are representing the number of proteins whose expression increases or decreases upon low temperature, short photoperiod or a combination of both factors exposure (Renaut et al. submitted).

Thus far, analysis of cold stress response using 2DE has been confined to organelles or specific tissues, for example, the nucleus of *Arabidopsis* (Bae et al. 2003), poplar leaves (Renaut et al. 2004) and *Arabidopsis* leaves (Kawamura and Uemura 2003), mitochondria of *Pisum sativum* (Taylor et al. 2005) and rice anthers (Imin et al. 2004). None of these studies, however, provided quantitative results from DIGE experiments. Generally speaking, initial proteomic studies have confirmed previously published data on cold-inducible proteins obtained from one-dimensional SDS-PAGE or gene expression studies. Several classes of cold-inducible or cold-repressible proteins have been identified using both approaches. Proteomics presents, however, major advantages linked to its high throughput capacity and its ability to perform simultaneously the analysis of hundreds of proteins from the same samples. A nonexhaustive list of common proteins detected by above cited 2DE during plant exposure to low temperatures is presented in Table 1.

Considered as a primary site of response to low temperature, a fraction of the plasma-membrane proteome of *Arabidopsis* has been characterized (Kawamura and Uemura 2003). Their results indicated a variation in the expression of 38 proteins, 27 of which were identified on a 2DE gel using a pH range of 4–7 (soluble fraction) and 15 proteins that were identified using a one-dimensional SDS-PAGE (insoluble proteins). The nuclear proteomic studies indicated that among the identified proteins using a pH 4–9 gradient, 40 were induced while 14 were repressed by cold treatment (Bae et al. 2003). In a proteomic study of pea (*P. sativum* L.) mitochondria, 33 proteins showed either up- or downregulation under different stress conditions, 20 of which appeared to respond to low temperature (4° C for 36 h) (Taylor et al. 2005). In rice anthers, a cold treatment at 12° C for 4 days induced differential expression of 70 proteins (12 new, 47 upregulated and 11 downregulated) of a pI between 4 and 7, with a positive identification for 18 of them (Imin et al. 2004). In leaves of poplar seedlings exposed to 4° C during 2 weeks, 26 proteins were identified using a 4–6 pH gradient that were COR, of which 21 were overexpressed and five

Table 1. Non-exhaustive list of common proteins detected by 2DE during plant exposure to low temperatures

Protein family	Variation of the expression	Tissue/cellular compartment/species	References
20S proteasome	Decrease	Bark/*Prunus*	Renaut et al. submitted
		Nucleus/*Arabidopsis*	Bae et al. 2003
2,3-bisphosphoglycerate independent phosphoglycerate mutase	Increase	Anther/*Oryza*	Imin et al. 2004
		Bark/*Prunus*	Renaut et al. submitted
Annexin	Increase	Plasma membrane/*Arabidopsis*	Kawamura and Uemura 2003
		Leaves/*Populus* hybrid	Renaut et al. 2004
Ascorbate peroxidase	Increase	Anther/*Oryza*	Imin et al. 2004
		Leaves/*Populus*	Renaut et al. 2004
Carbonic anhydrase	Increase	Mitochondria/leaves/*Pisum*	Taylor et al. 2005
		Plasma membrane/*Arabidopsis*	Kawamura and Uemura 2003
Chaperones	Increase	Mitochondria/leaves/*Pisum*	Taylor et al. 2005
		Leaves/*Populus* hybrid	Renaut et al. 2004
		Nucleus/*Arabidopsis*	Bae et al. 2003
		Bark/*Prunus*	Renaut et al. submitted
Dehydrins	Increase	Plasma membrane/*Arabidopsis*	Kawamura and Uemura 2003
		Leaves/*Populus* hybrid	Renaut et al. 2004
		Bark/*Prunus*	Renaut et al. submitted
HSP22	Increase	Mitochondria/leaves/*Pisum*	Taylor et al. 2005
		Leaves/*Populus* hybrid	Renaut et al. 2004
HSP70	Increase	Leaves/*Populus* hybrid	Renaut et al. 2004
		Bark/*Prunus*	Renaut et al. submitted
		Nucleus/*Arabidopsis*	Bae et al. 2003
		Mitochondria/leaves/*Pisum*	Taylor et al. 2005
		Anther/*Oryza*	Imin et al. 2004
HSP90	Increase	Nucleus/*Arabidopsis*	Taylor et al. 2005
		Mitochondria/leaves/*Pisum*	Bae et al. 2003
Malate dehydrogenase	Increase	Mitochondria/leaves/*Pisum*	Taylor et al. 2005
		Bark/*Prunus*	Renaut et al. submitted
Outer membrane lipoprotein	Increase	Plasma membrane/*Arabidopsis*	Kawamura and Uemura 2003
		Bark/*Prunus*	Renaut et al. submitted
Rubisco large subunit	Increase	Plasma membrane/*Arabidopsis*	Kawamura and Uemura 2003
		Leaves/*Populus* hybrid	Renaut et al. 2004
		Mitochondria/leaves/*Pisum*	Taylor et al. 2005
UDP-glucose pyrophosphorylase	Increase	Anther/*Oryza*	Imin et al. 2004
		Bark/*Prunus*	Renaut et al. submitted

repressed (Renaut et al. 2004). A brief discussion of the classes of proteins regulated by low temperatures is presented in following sections.

AFPs

AFPs have been shown to accumulate primarily in the apoplast under different stress such as cold, dehydration and short-daylength in both cereals and carrots (reviewed by Griffith and Yaish 2004, Moffat et al. in press). Their accumulation has been associated with an increase in the freezing tolerance of several plant species (Antikainen and Griffith 1997). It has been demonstrated that these proteins are involved in the lowering of the freezing temperature in cold-acclimated leaves and that after the leaves have frozen, they inhibit the recrystallization of intercellular ice (Griffith and Yaish 2004, Griffith et al. 2005). This implies that AFPs could prevent mechanical injury to cells by inhibiting both the size of individual ice crystals and the growth of crystals through intercellular spaces. Plant AFPs are homologous to pathogenesis-related (PR) proteins, e.g. β-1,3-glucanases, chitinases or thaumatin-like proteins (as reviewed by Griffith and Yaish 2004). Although AFPs are mainly extracellular in location and activity, Wisniewski et al. (1999) have documented that an intracellular peach dehydrin also had antifreeze activity. They suggested that intracellular AFPs may bind to intracellular ice nucleators and prevent intracellular ice formation (Wisniewski et al. 1999). In a proteomic

analysis of peach bark tissues using DIGE technology, one β-1,3-glucanase and one thaumatin-like protein accumulated in response to low temperature (Renaut et al. submitted).

Dehydrins and other LEA

LEA proteins are a family of proteins that were originally shown to accumulate in plant embryos during the later stages of embryogenesis (Dure 1993). Subsequently, they have also been shown to accumulate in response to low temperature, osmotic stress and abscisic acid (Wise and Tunnacliffe 2004). Several functional roles have been proposed for the different subgroups of LEA proteins including molecular chaperones, DNA binding and repair, as well as being a structural component of the cytoskeleton (as reviewed by Wise and Tunnacliffe 2004). Dehydrins, a subclass of LEA proteins, are heat-stable, glycine-rich proteins induced by a wide array of stimuli (drought, salinity, low temperature etc.) that result in dehydrative stress (Close 1996) and have been proposed to stabilize cell membranes and protect other proteins from denaturation when cellular water content is reduced during dehydration (Allagulova et al. 2003, Close 1996). This family of LEA proteins has been consistently reported to accumulate in response to low temperature in both herbaceous and woody plants (Wisniewski et al. 2004). This response has been also confirmed in proteomic studies dedicated to cold stress in plants where the expression of acidic dehydrins was shown to be induced in *Arabidopsis* (Kawamura and Uemura 2003, Renaut et al. 2004) and peach (Renaut et al. submitted), and the expression of one 10 kDa LEA in poplar was also documented (Renaut et al. 2004).

HSPs and chaperonins

All organisms respond to high temperatures by inducing the synthesis of a group of evolutionarily conserved polypeptides known as HSPs (Vierling 1991). When plants are exposed to high temperatures they synthesize both high molecular mass HSPs (from 60 to 110 kDa) and small HSPs (from 15 to 45 kDa). These proteins are also known as 'stress-related molecular chaperones' (Miernyk 1997). HSPs can also be induced by low temperatures and other environmental stresses such as drought, salinity or flooding (Sabehat et al. 1998). Families of HSP90, HSP70 and small HSPs have also been shown to accumulate in response to low temperature. Their accumulation in response to a wide array of environmental stresses is not surprising as these stresses can often result in protein dysfunction. HSPs and chaperonins are involved in several processes such as translation, translocation into organelles, refolding of stress-denatured proteins, prevention of aggregation of denatured proteins and membrane protection. HSPs and chaperonins often act in integrated fashion or as a complex to accomplish their functional role (e.g. HSP70 coupled with HSP40) (Miernyk 1997, Sung et al. 2001, Tsvetkova et al. 2002).

Accumulation of HSPs and chaperonins in response to low temperature has been demonstrated in different systems under cold stress, especially for the HSP90, HSP70 and small HSPs (Krishna et al. 1995, Lopez-Matas et al. 2004, Wisniewski et al. 1996). Similar results have also been obtained from cold stress studies using 2DE where both HSPs (HSP90 and HSP70; 22 and 20 kDa), as well as chaperonins (chaperonin 60 and 20), have been found to be induced by low temperature (Bae et al. 2003, Kawamura and Uemura 2003, Renaut et al. 2004, Taylor et al. 2005).

PR proteins

PR proteins are expressed in plants in response to pathogen infection, environmental stress, chemical compounds and wounding (van Loon 1997). They have also been shown to be expressed on a seasonal basis in the bark tissues of peach trees (Wisniewski et al. 2004). Fourteen groups of PR proteins have been identified, several of which are responsive to low temperature: PR-2 (β-1,3-glucanases), PR-3, PR-4, PR-8 and PR-11 (chitinases), PR-5 (thaumatin-like proteins) and PR-10 (Bet v-1 homologues) and PR-14 (lipid transfer proteins) (Griffith and Yaish 2004, Hincha et al. 1997, 2003, Kuwabara et al. 2002, Liu et al. 2003, Yeh et al. 2000). Although their role is not well understood, it has been suggested that they could be a component of the signal transduction pathway triggered during general stress response (Hoffmann-Sommergruber 2000). In peach bark tissue, different PR proteins (glucanases, PR-10 family, thaumatin-like protein) were identified as responsive to low temperature (Renaut et al. submitted).

Transduction, transcription and signalling pathways

The redox state of the cell can also be involved in the regulation of gene expression in response to low temperature through the involvement of H_2O_2 (Kocsy et al. 2001, Ensminger et al. this issue, Suzuki and Mittler, this issue). As H_2O_2 concentration increased in cold-exposed plants, it induced a change in the redox state. Higher concentrations of H_2O_2 led to increased activity of glutathione reductase and catalases among other

enzymes. Kocsy et al. (2001) have proposed that low-temperature-induced changes in the redox state as one of the signalling pathways triggered by low temperature that leads to changes in gene expression. Other signalling pathways are mediated by abscisic acid, Ca^{2+} or salicylic acid (Chinnusamy et al. this issue). Calcium serves the role as an important messenger in plant stress perception (White and Broadley 2003). Its cytosolic concentration will increase under cold exposure and transduces a signal via kinase and phosphorylation cascades (in particular MAPK- and CDPK-Ca^{2+}-dependent protein kinase). This signalling pathway is not only activated by cold but also by salinity, drought, hormones and pathogens (Knight and Knight 2001). Other proteins also bind Ca^{2+} during signal transduction, e.g. annexins (forming cation channel through the plasma membrane), calmodulins and calcineurins (White and Broadley 2003). It has been also shown that the Ca^{2+} influx in response to low temperature requires the re-organization of microfilaments of actin after rigidification of membranes has occurred (Orvar et al. 2000).

Among transcription factors, the family of C-repeat binding factors (CBF) have been demonstrated to activate cold responsive genes as well as genes involved in the dehydration response (Stockinger et al. 1997, Thomashow et al. 2001, Nakashima and Yamaguchi-Shinozaki, this issue). A transcription factor responsible for the induction of CBF gene expression has also been identified as ICE1 or inducer of CBF expression 1 during cold exposure (Chinnusamy et al. 2003, Van Buskirk and Thomashow, this issue).

In chilled poplar plantlets, CBF1, annexin and MAPK kinases were more abundant than in control plants (Renaut et al. 2004).

Enzymes

Changes in the expression and activity of enzymes involved in several different metabolic pathways have been shown to occur in response to low temperature (Guy 1990, Hurry et al. 1995). Among these, enzymes involved in carbohydrate metabolism (e.g. UDP-glucose pyrophosphorylase, 2,3-bisphosphoglycerate independent phosphoglycerate mutase, sucrose synthase, sucrose-phosphate synthase and invertase and enolase), in photosynthesis (e.g. rubisco subunits, rubisco activase and polypeptides of the photosystem II oxygen-evolving complex), in detoxifying enzymes (e.g. ascorbate peroxidase and superoxide dismutase), in proline metabolism (e.g. glutamine synthetase and proline dehydrogenase) and in lignin metabolism (e.g. caffeic acid 3-*O*-methyltransferase).

Several of these enzymes listed here have been shown to be regulated under low-temperature exposure in different 2DE studies (Imin et al. 2004, Kawamura and Uemura 2003, Renaut et al. submitted, 2004, Taylor et al. 2005).

Summary

Genomic technologies, such as high-throughput sequencing and microarrays, along with advances in bioinformatics have greatly increased our understanding of plant biology. More recently, advances in protein and metabolite technologies are now making global studies of these parameters more accessible. Integrating data from transcriptomics, proteomics, and metabolomics will allow for a more precise knowledge of how changes in gene expression lead to changes in metabolism. In particular, proteomics offers great potential for studying mechanisms of post-translational regulation as well as biosynthetic pathways.

Previous problems with the reliability and efficiency of separating proteins using 2DE have largely been overcome. Additionally, the use of DIGE technology now allows for rigorous statistical analyses of quantitative changes in protein abundance, and other techniques allow for identification of protein modifications, such as phosphorylation and glycosylation. Advances in spot picking and MS for identification of proteins have also made it possible to identify large numbers of proteins in a reliable manner. Collectively, these advances have enabled global studies of proteins to be conducted.

While constraints on the ability to extract and obtain a reasonable representation of the large number of proteins present in a cell at any given point in time still exists, proteomics offers the potential to integrate gene expression data with biochemical changes. The initial application of proteomics to low-temperature studies have generally confirmed an increased abundance in many of the classes of proteins, such a LEAs, antifreeze and PR proteins, that have been implicated in earlier studies. It is expected that the use of proteomics in the study of cold acclimation and the response of plants to freezing temperatures will greatly increase in the near future and provide a more comprehensive picture of how plants respond to their environment.

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